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Interactions between DNA and benzo- and tetrahydrobenzofurocoumarins: thermodynamic and molecular modeling studies

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Abstract

The non-covalent interaction of a series of new water-soluble benzo- and tetrahydrobenzofurocoumarins with salmon testes DNA has been studied using flow linear dichroism, circular dichroism, contact fluorescence energy transfer and ethidium bromide displacement assay. The new derivatives are characterised by having an alkyl amino side chain protonated at physiological pH; this fact strongly enhances the solubility in aqueous media and the affinity for the macromolecule. The results show significant difference in the affinity and the mode of binding among the examined compounds depending on the nature of the fourth condensed ring and the position of the alkylamino side chain. Benzofurocoumarins derivatives bind DNA by undergoing intercalation inside the duplex macromolecule, whereas tetrahydrobenzofurocoumarins derivatives show a substantial tilt relative to the base planes. Molecular modeling studies have been performed to characterise in detail the intercalation mechanism of these benzofurocoumarins to DNA.

Keywords: DNA binding; Linear dichroism; Benzofurocoumarins; Tetrahydrobenzofurocoumarins; Molecular modeling

1. Introduction

Furocoumarins are well-known compounds, which are extensively used as skin photosensitisers in the photochemotherapy of various skin diseases, such as psoriasis, vitiligo and mycosis fungoides [1]. Extracorporeal photochemotherapy (photopheresis) has been recently introduced in the treatment of autoimmune diseases such as T-cell lymphoma [2,3].

These compounds are also of interest because they are used as a probe in molecular biology and nucleic acid chemistry [4]. The photochemistry of furocoumarins has been extensively studied [5]. In the dark they are able to form a complex with base pairs of DNA in an intercalative manner, and upon exposure to near UV light (300–380 nm) to give a covalent addition to the 5,6 double bond of pyrimidine bases of DNA. The photoreaction leads to the formation of monoadducts or cross-links between the two strands of the nucleic acid due to the presence of two reactive sites in the furocoumarins moiety.

Although photochemotherapy has important therapeutic effectiveness, it is also accompanied by undesired side effects, both short (erythema, hyperpigmentation, genotoxicity) [6] and long term (cataract, risk of skin cancer) [7]. Cross-link formation is believed to be mainly responsible for the latter phenomena.

In order to obtain new monofunctional derivatives that have a good photoactivity with DNA but lack the undesirable side effects of phototoxicity, some tetracyclic compound, characterised by having a fourth aromatic or cyclohexenyl ring fused to the 4′,5′ position of the tricyclic furocoumarin structure have been synthesised and chemically characterised [8,9]. Moreover, the photoantiproliferative activity of tetrahydrobenzo- and benzofurocoumarins derivatives has been recently reported [9,10].

The present furocoumarin derivatives are positively charged due to a protonable side chain inserted at

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various positions of these compounds, in order to improve their very low water solubility. This fact strongly increases their affinity to the macromolecule and makes possible an accurate determination of their equilibrium constant.

The present report is concerned with spectroscopic and fluorimetric measurement accompanied by a conformational approach through a linear and circular dichroism study of the reversible interaction between DNA and the psoralen derivatives.

In order to understand further the binding modes suggested by the spectroscopic data, we carried out a computer modeling study using both molecular mechanics and flexible docking techniques.

The purpose of the present study is to better understand the mode of binding of these molecules in an attempt to gain insight about the local structure of the DNA-furocoumarin derivative complexes and its dependency upon the pattern of substitution.

2. Experimental protocols

2.1. Chemicals

Salmon testes DNA sodium salt ($\varepsilon_{260} = 6600\ M^{-1}\ cm^{-1}$), (Poly[dA-dT]$_2$ polydeoxyadenylic-thymidylic acid sodium salt ($\varepsilon_{260} = 6700\ M^{-1}\ cm^{-1}$), Poly[dG-dC]$_2$ polydeoxyguanylic-deoxycytidylic acid sodium salt ($\varepsilon_{260} = 7100\ M^{-1}\ cm^{-1}$), and ethidium bromide were purchased from SIGMA Chemical Co. and used without further purification. All other reagents were of analytical grade.

The aqueous buffer used was ETN buffer pH 7.0 (Tris 10 mM; EDTA 2 mM; and NaCl to adjust the ionic strength to 0.01 M). The synthesis and characterisation of the furocoumarins derivatives has been reported elsewhere [9,10].

2.2. Thermal transition studies

Absorbance temperature profiles were determined on a Perkin–Elmer 554 spectrophotometer with a temperature programmer. Samples were heated at a rate of 1 C° min^{-1} in a temperature range 25–100°C. Absorbance was monitored constantly at 260 nm. The melting profiles of DNA alone or drug–DNA complexes at a ratio [DNA]:[drug] = 10 in ETN 0.01 M buffer were analysed and the transition melting temperature T_m was determined from the first derivative plot of the melting curves. The ΔT_m values can be defined as the difference between for the DNA alone and DNA–drug complex.

2.3. Estimation of drug–DNA binding constant

Drug–DNA binding constants were estimated as described previously [11,12]. Briefly, 1 ml of ETN 0.01 M buffer (pH 7.0) was added to the appropriate DNA to give a final concentration of 1.26 μM in term of base pairs. The fluorescence was measured after equilibration of a few minutes using a LS –50B (Perkin–Elmer instruments) and an excitation wavelength of 525 nm and an emission wavelength of 595 nm. Aliquots of concentrated drug solutions (1–2 mg in 1 ml of DMSO) were added and the fluorescence was redetermined after mixing. Control experiments were performed to show that all compounds and their DNA-bound complexes showed neither optical absorbance nor fluorescence at 595 nm and there was no alteration of the fluorescence of unbound ethidium.

From a plot of the fluorescence versus added drug dose, the concentration of the drug required to reduce the fluorescence by 50% was determined (C_{50}) and used to calculate a relative binding constant.

The equilibrium binding constant for ethidium with salmon testes DNA was taken to be $9.5 \times 10^6\ M^{-1}$ bp [13] under similar conditions. The same K_{app} value for ethidium was assumed for the two polynucleotides on the basis of reported sequence-neutral binding behaviour with this ligand.

2.4. Circular dichroism

Circular dichroism (CD) is the differential absorption of circularly polarised light:

$$CD_{(\lambda)} = A_{L(\lambda)} - A_{R(\lambda)} \quad (1)$$

where $A_{L(\lambda)}$ and $A_{R(\lambda)}$ are the absorption spectra measured with left and right circularly polarised light. CD measurements were performed on a Jasco J500A spectropolarimeter equipped with an IBM PC and a Jasco J interface. Spectra were recorded in ETN (0.01 M, pH 7) at 25°C at different [DNA]:[drug] ratios.

2.5. Flow linear dichroism

Linear dichroism (LD) measurements were performed on a Jasco J500A spectropolarimeter equipped with an IBM PC and a Jasco J interface. Linear dichroism is defined as the differential absorption of orthogonal form of linearly polarised light:

$$LD = A_{\parallel} - A\perp \quad (2)$$

where $A_{\parallel}$ corresponds to the absorbance of the sample when the light is polarized parallel to the orientation of flow, and $A\perp$ is the perpendicular absorbance. By dividing other unoriented samples at rest with the absorbance, A_{iso}, the 'reduced' linear dichroism:

$$LD_r = LD/A_{iso} \quad (3)$$

is defined, which quantity may be related to the orientation of DNA (S) and the angle between the respective light-absorbing transition moment and DNA helix axis according to Norden et al. [14,15].

$$LD_r = 3/2 \times S(3\cos^2\alpha - 1) \quad (4)$$

The orientation is produced by a device designed by Wada and Kozawa [16] at a shear gradient of 700 rpm. Assuming a value of $\alpha = 90°$ for the DNA bases for a ligand bound to it, it follows that:

$$\alpha_L = \arccos[1/3 - (LD_r)_L/3(LD_r)_{DNA}]^{1/2} \quad (5)$$

where $(LD_r)_L$ is the reduced linear dichroism for the ligand, $(LD_r)_{DNA}$ is the reduced LD for DNA and α_L defines the ligand–DNA relative orientation. For the intercalated system, $\alpha_L \approx 90°$ and $(LD_r)_L \approx (LD_r)_{DNA}$.

2.6. Fluorescence contact energy transfer

The contact energy transfer from DNA bases and bound ligand was measured from the excitation spectra of the DNA–ligand complex in the wavelength range 220–330 nm, at an interval of 1 nm.

Excitation spectra were corrected for the inner filter effect prior to the normalisation [17]. The ratio $Q = q_b/q_f$, where q_b and q_f are the quantum efficiencies of bound and free ligand, respectively, was calculated for each wavelength using the equation: $Q = q_b/q_f = I_b E_f/I_f E_b$, where I_b and I_f are the fluorescence intensities of the ligand in the presence and in the absence of the DNA, respectively, and E_b and E_f are the corresponding ligand molar extinction coefficients. The ratio Q_λ/Q_{310} was then plotted against wavelength. The normalisation wavelength of 310 nm was chosen because of the negligible absorbance of DNA at this wavelength.

2.7. Computational methodologies

Calculations were performed on a Silicon Graphics O2 R10000 workstation.

This study involved the use of consensus dinucleotide intercalation geometries d(ApT) and d(GpC) initially obtained using NAMOT2 (Nucleic Acid MOdeling Tool, Los Alamos National Laboratory, Los Alamos New Mexico) [18] software. d(ApT) and d(GpC) intercalation sites were contained in the centre of a decanucleotide duplex of sequences d(5′-ATATA-3′)$_2$ and d(5′-GCGCG-3′)$_2$, respectively. Decamers in B-form were built using the 'DNA Builder' module of Molecular Operating Environment (MOE 1998.10) [19]. Decanucleotides were minimized using Amber94 all-atom force field [20], implemented by MOE modeling package, until the *rms* value of Truncated Newton method (TN) was < 0.001 kcal mol^{-1} per angstrom. The dielectric constant was assumed to be distance independent with a magnitude of 4.

The ground state geometries of all benzopsoralen derivatives were fully optimized without geometry constraints using RHF/AM1 semiempirical calculations [21]. Vibrational frequency analyses were used to characterize the minima stationary points (zero imaginary frequencies). The software package Gaussian98 was utilized for all quantum mechanical calculations [22].

Benzopsoralen **3**, **4**, and **6** were docked into both intercalation sites using flexible MOE-Dock methodology. The purpose of MOE-Dock is to search for favorable binding configurations between a small, flexible ligand and a rigid macromolecular target. Searching is conducted within a user-specified 3D docking box using simulated annealing and a molecular mechanics force field. MOE-Dock performs a user-specified number of independent docking runs and writes the resulting conformations and their energies to a molecular database file.

The resulting DNA–ligand intercalated complexes were subjected to Amber94 all-atom energy minimization until the *rms* of conjugate gradient was < 0.1 kcal mol^{-1} per angstrom. Charges for the ligands were imported from the Gaussian output files. The interaction energy values were calculated as the energy of the complex minus the energy of the ligand, minus the energy of DNA: $\Delta E_{inter} = E_{(complex)} - (E_{(L)} + E_{(rDNA)})$.

3. Results

3.1. Thermodynamic parameters

The examined compounds principally belong to two classes: benzo- and tetrahydrobenzofurocoumarins and their chemical structure are depicted in Fig. 1. They are substituted in various positions with dialkylaminopropoxylic or dialkylaminoamidic side chains, in which the basic nitrogen is protonated under physiological conditions (pK_a ca. 10.7). Moreover, the different positions of the substituent amino group could influences the orientation of the psoralen moiety relative to the DNA helix axis.

Two independent techniques were used to assess the DNA binding properties of furocoumarins derivatives with DNA in aqueous solution. First, relative binding-behaviour was determined from the drug induced thermal stabilisation of the double stranded DNA by each ligand compound at fixed [DNA]:[drug] molar ratio 10:1. In the second method, apparent K_{app} binding constant values were determined using a competitive fluorometric ethidium displacement method that has been extensively used for other DNA binding ligands, in particular intercalants [12,13]. On these bases, the

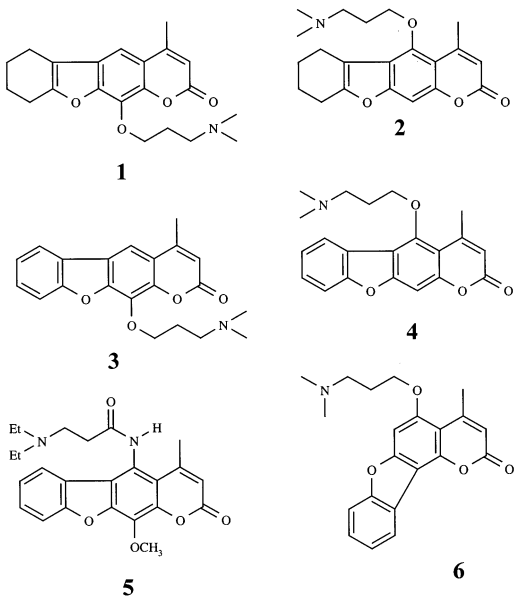

Fig. 1. Chemical structure of the examined compounds.

data presented in Table 1 can be regarded as indicative of the strength and extent of binding to the macromolecule.

The results indicated a significant stabilisation of the macromolecule except for compound **5**. In particular, the angular compound **6** (benzoangelicin) presents the highest value of ΔT_m. It should be stressed that ΔT_m values can provide only a qualitative ranking order for DNA given the thermodynamic basis of this technique, but can nevertheless be used to rank series of structurally related compounds.

Another technique useful to evaluate the relative affinity of intercalating molecules is ethidium displacement assay. The C_{50} is the ligand concentration required to reduce the fluorescence of the ethidium–DNA complex by 50% and used for the calculation of K_{app}.

According to the melting studies, compounds **2**, **3**, **4** and **6** show a considerable affinity toward the DNA. In any case, the presence of a basic side chain strongly enhances the binding affinity toward the nucleic acid compared with the parent compound benzopsoralen [23].

Further ethidium experiments were conducted for compound **3** and its angular isomer **6** to verify their potential preference toward specific sequence utilising two synthetic polynucleotides (Poly[dA-dT]$_2$ and Poly[dG-dC]$_2$). The obtained results indicated a marked preference of these derivatives, in particular compound **6** toward A-T sequences the value of the ratio being K_{AT}:K_{GC} 2.4 and 5.2 for **3** and **6**, respectively.

3.2. Flow linear dichroism

The LD spectra of DNA complexes of all the test molecules are shown in Fig. 2. The LD signals in the ligand absorption regions (300–380 nm) are negative indicating an induced orientation of the ligand chromophore upon binding to the DNA. A significant increase in LD values in the DNA absorption band (260 nm) was observed, as shown in Fig. 2, suggesting that the ability of the DNA molecules to orient the flow lines is increased upon binding to these compounds. The increase observed in the LD spectrum of the DNA upon complexation is indicative of the stiffening of the DNA double helix upon binding, allowing for a better alignment of the DNA molecule within the cuvette cell.

According to the Eq. (5) (see experimental protocols), we were able to evaluate the angle α between the respective light absorbing transition moment and the DNA helix axis. It can be observed in Table 2 that among the compounds studied only compounds **3**, **4**, and **6** bind to the DNA by perfect intercalation. It can also be noted that for compounds **3** and **4**, the negative LD_r amplitudes are found to be larger in the chromophore absorption region than in the DNA band. It should be emphasised that the proposed model (Eq. (4),

Table 1
Thermodynamic parameters for the binding of the test compounds in ETN 0.01 M buffer pH 7.0

Comp.	1	2	3	4	5	6
ΔT_m [a]	3.5 ± 0.7	4.1 ± 0.2	4.2 ± 0.5	7.2 ± 03	1.2 ± 0.5	11 ± 0.6
C_{50} [b]	358.4 ± 67.3 [e]	14.57 ± 0.22	23.92 ± 0.18	7.83 ± 1.32	1036 ± 48 [e]	1.66 ± 0.03
C_{50} Poly[dA-dT]$_2$ [c]	n.d.	n.d.	10.39 ± 0.36	n.d.	n.d.	0.55 ± 0.04
C_{50} Poly[dG-dC]$_2$ [c]	n.d.	n.d.	24.62 ± 0.32	n.d.	n.d.	2.85 ± 0.05
K_{app} ($\times 10^{-5}$ M^{-1} bp) [d]	0.33 ± 0.061	8.21 ± 0.12	5.00 ± 0.037	15.3 ± 2.57	0.11 ± 0.005	72.1 ± 1.30

[a] ΔT_m values (°C) determined at a binding ratio [DNA]:[drug] = 10; mean ± SEM from three determinations.

[b] Concentration (μM) of drug needed to reduce the ethidium–salmon testes DNA complex fluorescence by 50%; mean ± SEM from three determinations.

[c] As in [b] determined with synthetic polynucleotides

[d] Apparent binding constant for the binding of test compounds with salmon testes DNA, defined by $K_{app} = K_{et} \times ([EtBr]/[C_{50}])$, where $K_{et} = 9.5 \times 10^6$ M^{-1} bp.

[e] Values extrapolated from the linear portion of the curves.

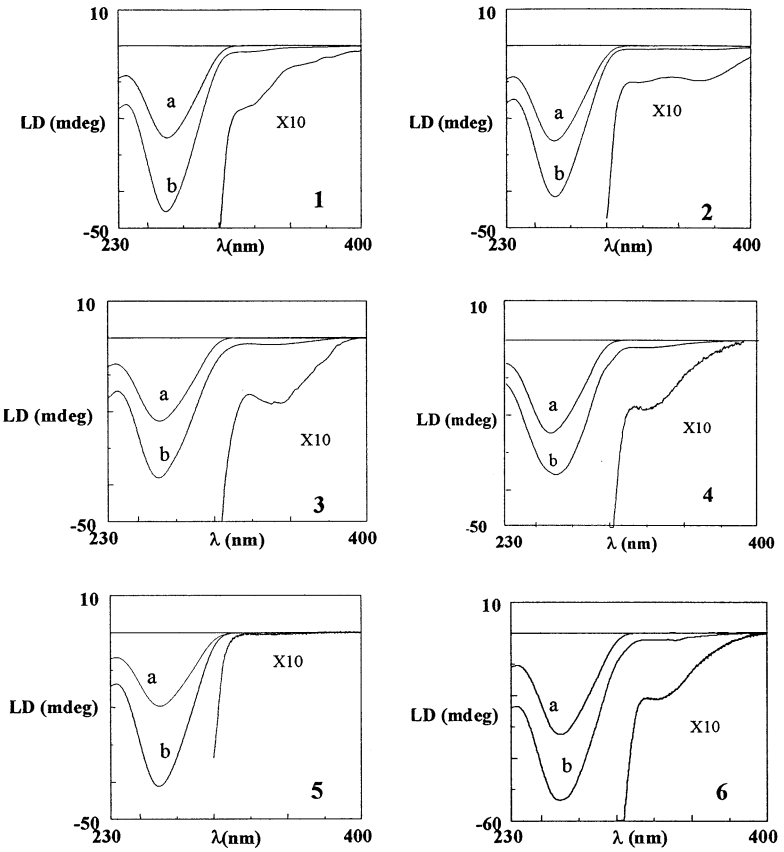

Fig. 2. Flow linear dichroism spectra of the DNA complexes of the examined compounds at the molar binding ratio [DNA]:[drug] = 12.5. The symbol a-b refer to DNA alone and DNA-drug complex, respectively.

see experimental protocols) does not exclude the possibility of isotropic deviation, either static or dynamic, of the DNA base planes from the perpendicular orientation, in fact the DNA bases can be inclined by as much as 20° from the helix axis [24]. Thus, a higher LD_r amplitude than for the DNA bases has in many cases been observed for many intercalators including ethidium and acridines [25], ruthenium polpyridil complexes [26], ellipticine derivatives [27] and the dyes cianine oxazole yellow and methilene blue [28,29], and implies that on average the bound ligand molecules are oriented more perpendicular to the helix axis than the DNA bases. This suggests that there may be either significant inclination of the bases from perpendicular orientation and that compounds **3** and **4** are intercalated with their transition moments parallel to the tilt axis or local stiffening of the helix at the intercalation sites.

In contrast a non perfect intercalative DNA binding is indicated for compounds **1**, **2**, and **5**. For the last compound it binds to the DNA at an angle close to the pitch of minor groove, a geometry that excludes intercalation.

3.3. Circular dichroism

Circular dichroism is defined as the difference between the absorbance measured with left and right

Table 2
Average angle α_L between major transition moment in the molecular plane of the drug and the overall orientation (helix) axis of the DNA [a]

	λ (nm)	LD_r	α_L (°)
DNA	260	−0.0267	
Compound **1**	326	−0.0197	73
DNA	260	−0.0249	
Compound **2**	370	−0.0216	78
DNA	260	−0.0276	
Compound **3**	340	−0.0303	90
DNA	260	−0.0224	
Compound **4**	320	−0.0271	90
DNA	260	−0.0218	
Compound **5**	320	−0.0095	64
DNA	260	−0.0260	
Compound **6**	330	−0.0260	90

[a] The values of angles were evaluated from LD_r data at the indicated wavelength and are representative of two experiments.

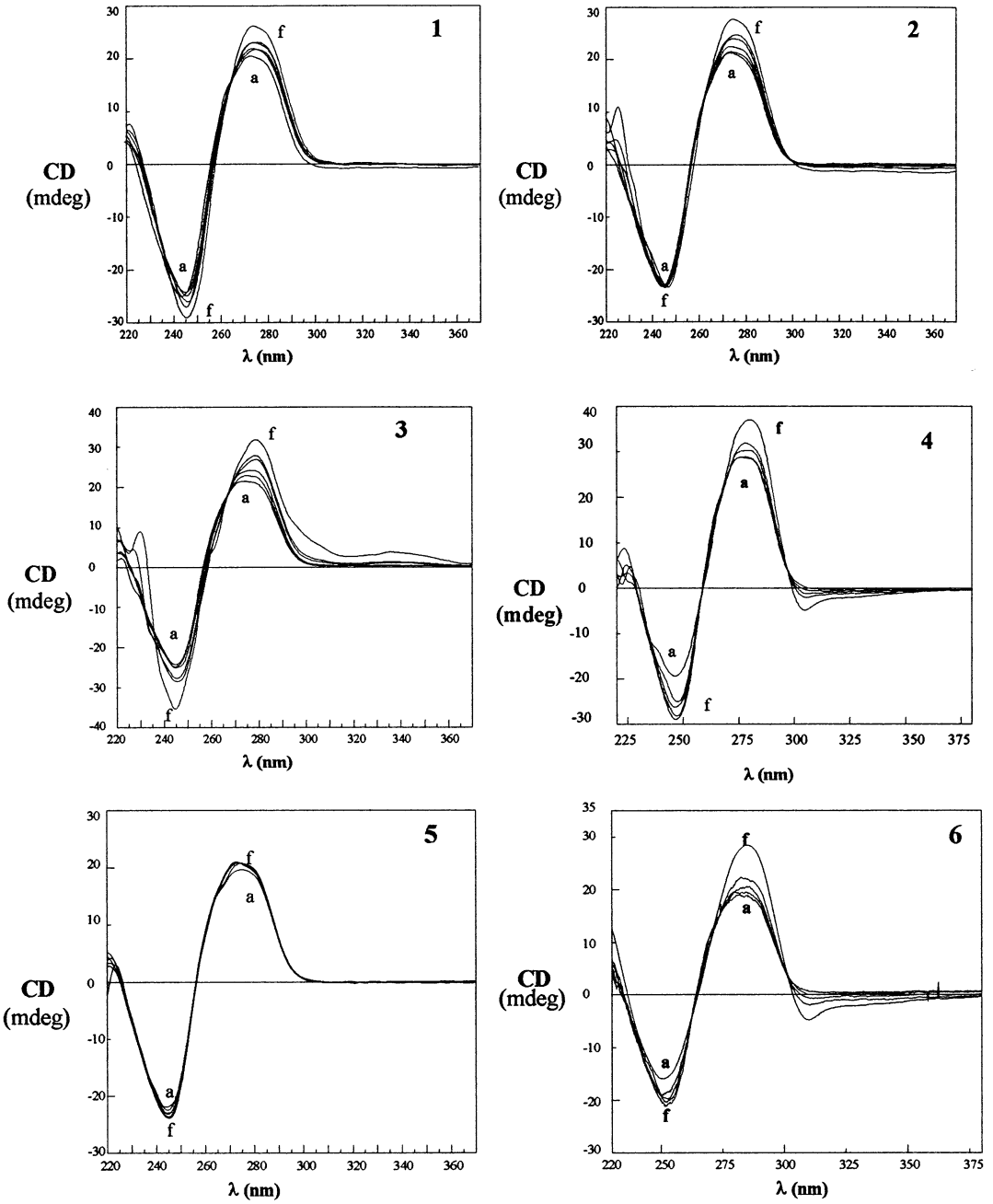

Fig. 3. Circular dichroism spectra for examined compound complexes with DNA. The symbol a-f refer to increasing [DNA]:[drug] ratios $R = 0$, 50, 25, 12.5, 10, 5, respectively.

hand circularly polarised light. The CD spectrum spectra of the ligand–DNA complexes can provide information at two levels. First, the conformation of polynucleotide itself can be probed through the CD of the intrinsic DNA absorption near 260 nm. Second, although the drugs are all achiral molecules, they acquire an induced CD signal when they form complexes with the macromolecule. The CD is induced by the interaction between the bound ligand and chirally arranged base transitions and is dependent upon the position and the orientation with respect to the polynucleotide bases [30,31].

Fig. 3 shows the circular dichroism spectra of the examined compounds. It can be observed that for all examined compounds except **5**, a significant perturbation of the DNA structure occurs as evidenced by the increase of the positive band of DNA at 275 nm upon binding of increased amount of ligand to the DNA. In the chromophore absorption region an induced positive dichroic signal can be observed for compound **3**,

whereas a negative one can be observed for compounds **4** and **6**. In contrast, no induced CD can be observed for the other molecules examined.

3.4. Fluorescence energy transfer

In addition to linear dichroism, another method by which the binding mode of a ligand may be analysed involves the use of the fluorescence energy transfer experiments, which probe for the transfer of the excitation energy from the DNA bases to the ligand. Excitation energy transfer exhibits a distance dependence of r^{-6}, where r is the distance between the species involved in the transfer of energy . This distance dependence, coupled with the low quantum yield of the DNA bases [32], gives rise to short (4–7 Å) Förster critical distance R [33], at which half of the energy absorbed by the DNA bases is transferred to the bound drug. Drug insertion between stacked bases within a host duplex (intercalation) is associated with short base-to-drug distances (about 2–4 Å) and the presence of base–drug stacking interactions.

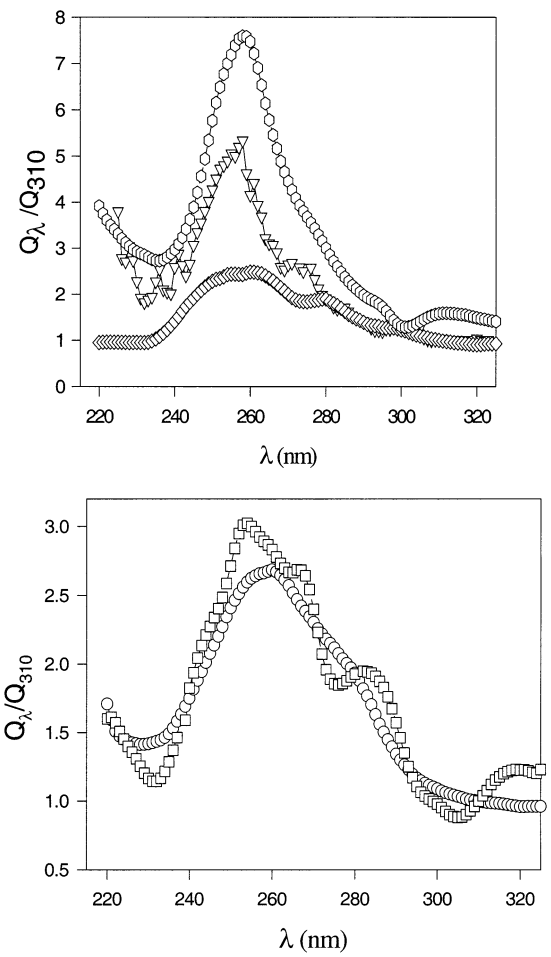

Fig. 4. Fluorescence energy transfer from DNA bases and bound compounds (□ = compound **1**, ◇ = compound **2**, ○ = compound **3**, ⬡ = compound 4, ▽ = ethidium bromide). The [DNA]:[drug] ratio was ten for all compounds except for ethidium bromide (ratio = 28). Experiments were carried in ETN 0.01 M buffer at 25°C.

Thus, the observation of energy transfer from a host DNA to a bound drug is consistent with an intercalative mode of binding. In contrast, drug binding to the minor groove and/or drug stacking along the surface of the helix are associated with longer to base-to-drug distances and minimal base-drug stacking interactions. Consequently, such binding modes are consistent with the absence of energy transfer from the host DNA to a bound drug.

Fig. 4 shows the curves depicting the wavelength dependence of Q_λ/Q_{310}, the relative quantum yield of the complexes of compounds **1**, **2**, **3** and **4** with salmon testes DNA at a molar ratio [DNA]:[drug] = 10, versus free drug, normalised with respect to 310 nm, a wavelength at which the DNA bases do not absorb. As a positive control a similar curve for the 'classical' intercalator ethidium bromide at a molar ratio [DNA]:[drug] = 28 is also presented.

These curves reflect the difference between the fluorescence excitation and the absorbance spectra of the free drug and those of the DNA-bound drug. It can be observed that these differences are significative below 300 nm, reaching the maxima values at wavelengths corresponding to the absorption maxima of the DNA (between 255 and 260 nm). The observation of energy transfer from the DNA bases to bound ligands thus provide strong evidence for intercalative mode of binding of these benzofurocoumarins derivatives.

3.5. Molecular modeling

Modeling studies have been previously performed to characterise the intercalative binding of psoralen and angelicin derivatives to DNA. In the present study, we have analysed in detail the intercalation properties of three structurally different benzofurocoumarins to better define the steric and electronic requirements during the intercalation process. In Fig. 5 we report the optimized structures of benzofurocoumarins **3**, **4**, and **6**.

Flexible docking experiments were performed to calculate the energy changes and optimal geometries corresponding to the intercalative binding of benzofurocoumarins derivatives to A-T and G-C steps in a double helical structure. In agreement with the binding experiments reported above, only alternating purine-pyrimidine steps were considered. The docking data show benzofurocoumarins to adopt two main binding modes, one intercalative and the other a binding mode involving the interaction in the DNA minor groove. However, comparing the theoretical binding energy obtained after the flexible docking analysis, it seems to confirm that the intercalative process is energetically favoured.

Another interesting consideration is that the energy balance for the most favourable intercalation of the **6** derivative confirms a preference for A-T steps (− 54.3

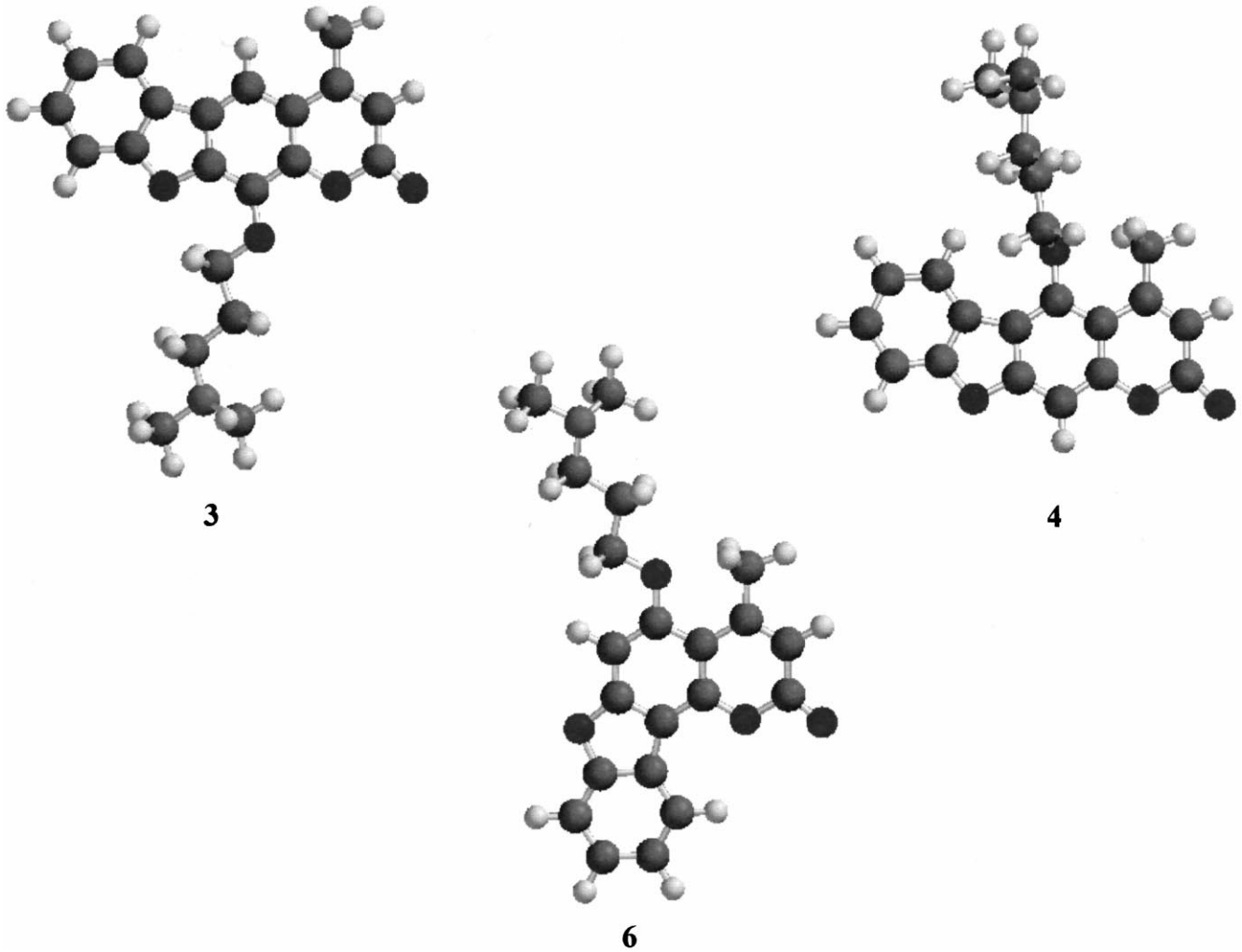

Fig. 5. RHF/AM1 optimized structures of benzosporalens **3**, **4**, and **6**.

kJ/mol) versus G-C steps (-42.9 kJ/mol). Similar results have been obtained for the other derivatives. The results are presented in Fig. 6. The best binding configuration of the **6** derivative locates the side chain in the major groove. The orientation of the intercalated chromophore is quite similar to that proposed for other furocoumarins, as it exhibits a predominant component in the direction parallel to the base pair longest dimension. The intercalation geometry is in accord with the well-known photoreactivity of psoralen derivatives with the DNA structure. In fact, after the intercalation process the cycloaddition reaction generally occurred between the furan-side or pirone-side double bond of furocoumarin moiety and the 5,6 double bond of a thymine ring. This photoreactivity is generally described as a [2 + 2] photocycloaddition reaction with the pyridine bases.

Hence, the theoretical computations, supported by experimental evidence, suggest the preference of the drugs for alternating purine-pyrimidine site, the most effective consensus sequences containing AT sites. The preferred location of side-chains was found to be in the major groove. We are tempted to conclude that a very similar mode of binding is generally operating in this class of benzofurocoumarins.

4. Discussion

It is well known that furocoumarins interact with the DNA in a manner very similar to that of other polycyclic aromatic compounds, which are able to intercalate between two adjacent base pair of the double helix, forming a reversible molecular complex. After irradiation with near UV light the intercalated psoralen molecule can form a covalent conjugate with nearby pyrimidine bases of DNA.

In order to establish a relationship between the binding mode and the photobiological activity, we have performed a study to better characterise the structure of furocoumarins derivatives–DNA complexes. As is well known the final photocycloaddition process depends on

a proper orientation of the molecules between two adjacent pyrimidine bases.

As already reported in the results section, dichroism and molecular modeling studies shown that the benzofurocoumarins interact with DNA through an intercalative mechanism. The site selectivity of these derivatives for the AT site is explained by their ability to adapt to the base pair propeller twist of DNA to optimise stacking and the hydrophobic interaction between the thymidine methyl group and the planar benzofurocoumarins system. The alkylamino side chain is located in the major groove of DNA.

The present binding data show significant differences among the examined compounds, depending on the nature of the fourth condensed ring (aromatic or cyclohexenyl), and on the position of the alkyl amino side chain. Generally the presence of cyclohexenyl ring reduces binding affinities compared with the corresponding aromatic derivatives. As reported in Tables 1 and 2, compounds **1** and **2** are less active compared with **3** and **4** derivatives. This is probably due to the steric hindrance of the non-planar cyclohexenyl ring. These effects are as previously observed with other tetrahydro benzofurocoumarins derivatives [23]. Molecular modeling data are consistent with experimental results. In fact, the intercalation energy of **1** to the AT base pair is 13 kJ mol^{-1} less stable than **3**.

The presence of a lateral side chain inserted in position 5 remarkably increased the affinity to the macromolecule for all the studied benzofurocoumarins. This observation is confirmed by the fact that tricyclic derivatives, such as 5-methoxypsoralen and the 5(diethylaminopropiloxy)psoralen, also exhibit binding constants three and seven times higher than the analogues isomer substituted at position 8 [34,35]. As shown in Fig. 6, the alkylamino side chain is located in the major groove of DNA and it can interact, through hydrogen bonding, with the NH_2 of the adenine moiety of the intercalative site bases.

The angular derivative **6** show, in comparison to the linear compounds, the highest affinity for DNA (ten times more in comparison with the linear isomer **3**). For compound **6** and its linear derivative (isomer **3**) a marked preference toward the A-T sequence has been demonstrated, unusual for classical intercalators [36,37]. As shown in Fig. 6, three key features might characterise this favourable stacking interaction: (i) near perfect parallel alignment between the sandwiched AT bases and polycyclic aromatic system; (ii) substantial areas of overlap in the two-stacked rings, and (iii) a 3.4 Å interplanar spacing within the overlapping region. It is also interesting to note that different orientation of the compounds can be observed with respect to

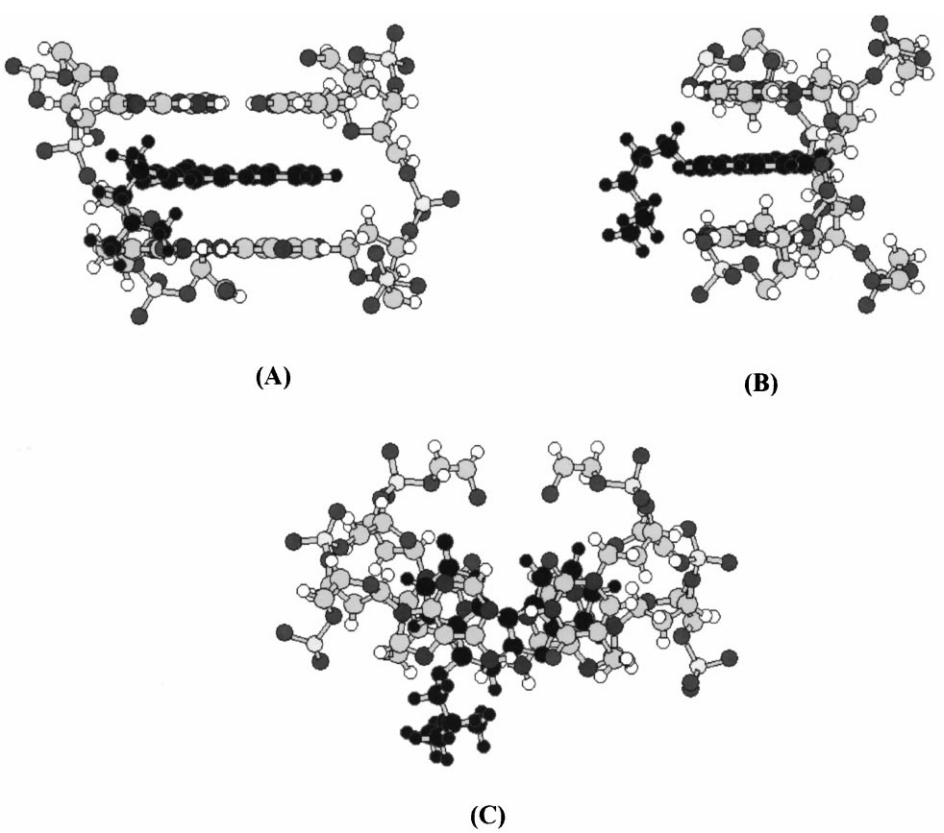

Fig. 6. Intercalation site of the **6**-oligonucleotide complex viewed in a projection plane parallel to the helix axis (A and B) and viewed in a projection plane orthogonal to the helix axis (C).

the pattern of substitution and the nature of the chromophore, as observed for other furocoumarin derivatives. The LD results give direct evidence of the geometry of the complex; tetrahydrobenzofurocoumarins derivatives show a considerable tilt relative to the average orientation of the plane of base pairs. In contrast, benzofurocoumarins derivatives show angle values of 90° as expected for a perfect intercalator. Compound **5**, which is a disubstituted compound, is an exception in this series, with significantly lower values of α and also lower interaction efficiency. These facts are probably due to a combined effect of the two substituents and are in agreement with a previous study performed with 5-substituted 8-methoxypsoralen [38].

The circular dichroism results support very well the data obtained with linear dichroism. As observed in Fig. 3, tetrahydro derivatives **1** and **2** have an effect only on the DNA circular dichroism spectrum upon binding to the macromolecule. Furthermore, the absence of induced dichroic signal in the chromophore absorption region indicates that they are not sufficiently close to the DNA bases for these to induce any signal.

A different behaviour is observed upon DNA interaction for the benzo-derivatives for which an intercalative binding to the DNA has been demonstrated. For this series of compounds we observe a relatively strong perturbation of the DNA circular dichroism spectrum, and also a significant induced CD signal in the chromophore absorption region when they bind to the DNA. The induced CD signals of intercalated molecules have been correlated to the relative orientation of the ligand molecule and base pairs. However, it has been shown that the induction signal depends not only upon orientation but also upon sequence context and displacement of the intercalated molecule, with reference to the base pair vertical axis [39,40].

Further evidence for intercalation can be obtained from the fluorescence studies using the fluorescence contact energy transfer method. Contact energy transfer is a useful tool that effectively distinguishes between intercalation and groove or externally binding mode [41], as demonstrated in several studies [42,43].

Fluorescence energy transfer occurs only if the bound drug is in close proximity to and in correct orientation with the base as in the case of intercalation. Molecules that are bound on the surface of the helix due to electrostatic interactions are not situated correctly for efficient energy transfer through the bases. The increase in the relative quantum yield at about 260 nm for these derivatives clearly demonstrated the presence of energy transfer in the region of absorbance of DNA bases.

In conclusion, the results obtained in this study, from several physicochemical techniques, have provided detailed information concerning the possible mechanism of DNA binding of some new water-soluble furocoumarins. Moreover, an interesting correlation can be established between the molecular modeling analysis and the binding data and this approach can be used as a tool for a better understanding of the effects of various ligands on DNA conformation upon DNA binding.

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