Flavonoid–DNA Interaction Studied with Flow Linear Dichroism Technique

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Quercetin and other flavonoids which had been previously found to be mutagenic in the Ames test, have been proved to inhibit tumor development in several experimental animal models. We studied the interaction between DNA and a series of flavonoids whose biological activity was known to span a wide range of potency: quercetin (the most active), morin, rutin, naringin, and 2,3-dihydroquercetin (inactive). The sensitivity of equilibrium binding analysis carried out by the dextran–polyethylene glycol–H_2O biphase system was not high enough to reveal and evaluate the binding affinity of these flavonoids toward DNA. The much more sensitive flow linear dichroism technique provided evidence that they can bind DNA by intercalation and that their affinity for DNA increased following the same sequence of their biological activity.

Keywords: *Flavonoids; DNA; flow linear dichroism; flavonoid–DNA interaction*

INTRODUCTION

The flavonoids are universally present in vascular plants. Fruits and vegetables contain so much flavonoids that human daily intake of these compounds was estimated to vary from 50 mg to 1 g (Kühnau, 1976).

Since the Ames *Salmonella typhimurium* mutagenesis test was described (Ames et al., 1975), many laboratories have tested the mutagenecity of flavonoids in this *in vitro* test system. Several of them, most notably quercetin, one of the most widespread in human dietary vegetables and fruits have been found to be mutagenic (Bjeldanes and Chang, 1977; Brown et al., 1979; Brown and Dietrich, 1977; Hardigree and Epler, 1978; MacGregor and Jurd, 1978). These results attracted public attention and concern. Systematic studies were therefore undertaken also *in vivo* and no clear-cut evidence was obtained that they can be carcinogenic (McGregor et al., 1983; Wargovich and Newmark, 1983).

Moreover, the same flavonoids which had been previously found to be mutagenic in Ames test have been proved to inhibit tumor development in several experimental animal models (Huang and Ferraro, 1992). Quercetin in particular was found to be a potent anticarcinogen against skin, colon, and mammary cancers in rodents (Deschner, 1992; Leighton et al., 1992; Verma, 1992; Weisburger, 1992; Yasukawa et al., 1988). Quercetin can also inhibit the induction and progression of human cancers (Leighton et al., 1992; Verma, 1992; Yoshida et al., 1990).

These conflicting biochemical activities and the fact that the most potent anticancer derivative is also apparently the most mutagenic one is very puzzling. It was suggested that the phenols could apparently test positive as mutagen because of artifactual errors due to the test conditions (Newmark, 1992). It is well known that *o*-dihydroxy phenols, like quercetin, can readily oxidize. The highly aerobic conditions of the Ames test and trace metals, like copper or iron, which are potent catalysts for the oxidation of phenols, could lead to *in situ* production of hydrogen peroxide via an intermediate superoxide and thus cause DNA strand breakage.

The flavonoid anticancer activity was associated with a large variety of mechanisms (Huang and Ferraro, 1992). Among them, the ability of flavonoids to act as scavenger of superoxide anions was suggested to play a very important role. On the other hand it is well established that DNA is the main target for both chemical carcinogens (Blackburn and Kellard, 1986) and anticancer drugs (Douglas, 1984). Flavonoid–DNA interaction has never been taken into account for flavonoid biological activities because no evidence of their complexes with DNA were made available so far, as pointed out by Huang and Ferraro (1992).

By the flow linear dichroism (LD) technique we studied possible interaction between DNA and a series of flavonoids whose biological activity was know to span the full range of potency: from quercetin **(a)** the most active, to dihydroquercetin **(d)** which is inactive (Huang and Ferraro, 1992). We obtained evidence that biochemically active flavonoids can bind DNA, by intercalation, and that their affinity for DNA basically follows the same sequence of the potency of their activity.

MATERIALS AND METHODS

Quercetin **(a)**, rutin **(a')**, morin **(b)**, and naringin **(c)** (HPLC grade) were purchased from Extrasynthese (Gemay, France) and dihydroquercetin **(d)** (taxifolin) was purchased from Sigma Chemical Co. and used without further purification. Highly polymerized calf thymus DNA (Sigma) was repeatedly deproteinized (Maniatis et al., 1982) until a ratio A_{260}/A_{280} ranging between 1.80 and 1.88 was reached.

Flow Linear Dichroism. The LD spectra were recorded by a JASCO J500 dichrograph with an LD attachment and evaluated by the approach described by Samorì (1983). The

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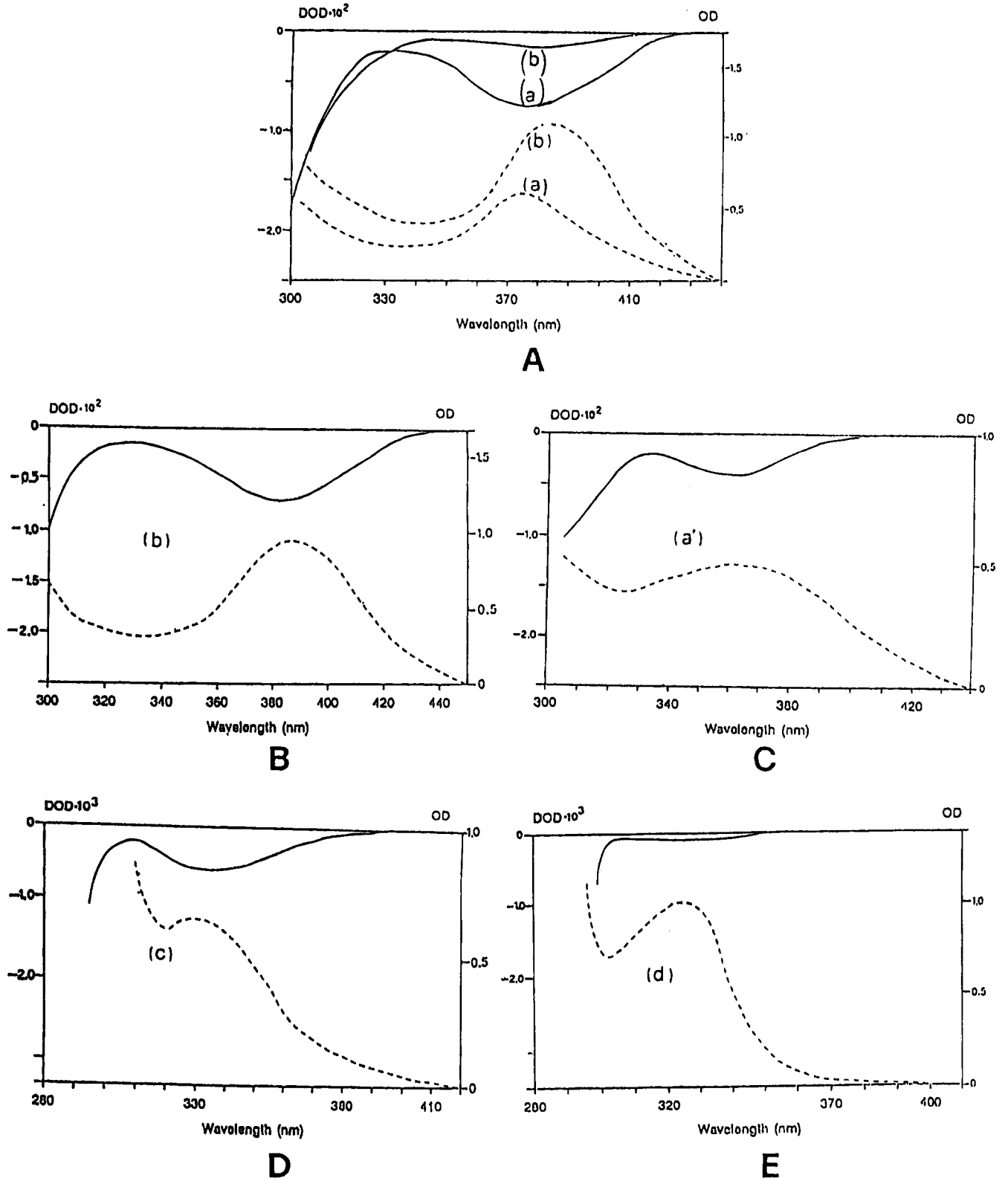


Figure 1. Flow LD (—) and average absorption spectra (- - -) of (A) quercetin (**a**) and morin (**b**), (B) morin (**b**), (C) rutin (**a'**), (D) naringin (**c**), and (E) dihydroquercetin (**d**). The A spectra were recorded in buffer (TRIS 100 mM, NaCl 200 mM, pH = 7.5)–EtOH (30%); spectra B–D, in the same buffer without ethanol additon.

DNA flow orientation was assured by a cylindrical cell based on the design of Wada (1964). The cell was built in the machine shop of the Faculty of Industrial Chemistry of Bologna. It contains two transparent concentric cylinders of fused quartz made for us by Hellma (Müllheim). The outer cylinder is stationary, the inner can rotate at variable speed thus producing a shear in the DNA solution contained in the annular gap. The LD spectra were recorded at a shear rate (Norden et al., 1992) of

$$G = \omega R_o/R_o - R_i = 3600\ s^{-1}$$

where $\omega = 2\pi n/60$ is the angular velocity, n is the number of revolutions per minute, and R_i and R_o are the inner and outer cylinder radius, respectively. DNA concentration was 10 mg/mL when the lowest energy flavonoid band (in the 320–380 nm region) was investigated (Figure 1) or 0.5 mg/mL when the LD of the DNA band centered at 260 nm was recorded, instead.

Molecular Orbital (MO) Calculations. The CNDO/S method was implemented by a configuration interaction of selected singly and doubly excited configurations. The calculations were carried out with 110 configurations, using the Ohno–Klopmans approximation for the Coulomb integrals (Ohno, 1964). The molecular conformation and the atom coordinates were obtained by Alchemy II (Tripos Associates Inc., St. Louis, MO, 1989).

Liquid Crystal Linear Dichroism. ZLI 1167 (Merck, Darmastad) was used as thermotropic liquid crystal (Samorì and Mattivi, 1986; Laurent and Samorì, 1987).

FLOW LINEAR DICHROISM TECHNIQUE

The intensity of the absorption of plane-polarized light by an oriented (linearly anisotropic) sample depends upon the orientation of the sample optical axis with respect to the direction of polarization of the light. Absorptions can take place only if some collinearity exists between the transition moments j which can be generated in the sample and the direction of polarization of the light. If two plane-polarized components of the incoming light are sent to the oriented sample with the directions of polarization parallel (||) and perpendicular ($\perp$) to its optical axis, their absorptions will therefore be different. The difference between the two optical densities, $DOD = (OD_{||} - OD_{\perp})$ i.e. the linear dichroism (LD), is linked to an order parameter S_{jj} which depends upon the distribution of the orientations of the molecules in the sample

$$LDA/A_{is} = (OD_{||} - OD_{\perp})/(OD_{||} + 2OD_{\perp}) = 3S_{jj} \quad (1)$$

where $A_{is} = (OD_{||} + 2OD_{\perp})$ is the average absorption.

The LD is therefore a source of information about the orientations of the molecules in the sample, provided that we know: (i) the direction in the molecular framework of the transition moment j responsible of the light absorption, (ii) the orientation of the sample optical axis with respect to the light polarization direction.

Linear Dichroism of DNA. The macroscopic sample orientation required to run LD spectra of DNA is easily achieved by subjecting its concentrated solution to a shear gradient (Norden et al., 1992). If the LD is recorded by a phase modulation technique the available sensitivity is improved by several orders of magnitude (see Materials and Methods). This allows studies at the lowest possible ligand–DNA binding ratios. When a DNA solution is oriented in a cylindrical flow cell (see Materials and Methods) the DNA chains become preferentially aligned to the shear flow whose direction coincides with the sample optical axis. The chromophores responsible of the lowest energy (near-UV) absorption band are the DNA bases and the transition moment of this band, centered at 260 nm, is on the plane of the bases, i.e. perpendicular to the optical axis. The flow oriented sample is rotated in order to have its optical axis parallel to one component of the modulated sequence of the two perpendicularly polarized light components. The light component whose polarization is perpendicular to the sample optical axis will thus be preferentially absorbed. A negative band of LD (in eq 1 $OD_{||} < OD_{\perp}$) will thus be recorded, and its intensity and profile provide information about the order parameter of the DNA axis (S_a).

Linear Dichroism of DNA–Ligand Complexes. After having recorded the negative linear dichroism band at 260 nm of the DNA the ligand under investigation is added to the DNA solution. The LD spectrum is recorded again extending the spectral range of investigation to the region of the ligand absorption bands. Two possibilities are open:

(a) *The Ligand Does Not Bind to DNA.* It cannot therefore assume any preferential orientation with respect to the planes of polarization of the incoming light. The flow alone is not able to orient a nonpolymeric molecule. No LD signal is therefore exhibited by the ligand molecule.

(b) *The Ligand Does Bind to DNA-Oriented Chains.* The DNA orientation is transferred to the ligand and LD signals are recorded in correspondence of its absorption band.

This method makes it possible to detect, with very high sensitivity, any binding affinity of the ligand toward DNA. From the sign of the LD signal we can usually identify, very directly, the geometry of the complex also. In fact whenever the ligand chromophore intercalates between the DNA base pairs and has transition moments on its molecular plane, a negative LD signal is expected. In fact in this case the transition moments of the bound ligand are preferentially perpendicular to the shear direction. We will see that this is what is taking place when flavonoids bind DNA and negative flow LD signals are recorded in correspondence of both DNA and flavonoid absorption bands.

The order parameter S_{jj} of the transition moment j with respect to the flow direction is experimentally obtained by eq 1. The LD signal is the result of both the orientation of the DNA chains with respect to the flow direction (S_a) and the orientation of the bound ligand molecular axes with respect to the axis of the host DNA chains (S'_j). This means that the order parameters S_{jj} can be factorized into S_a and S'_j (Norden et al., 1992; Forni et al., 1989; Samorì, 1988b):

$$S_{jj} = S_a S'_j \quad (2)$$

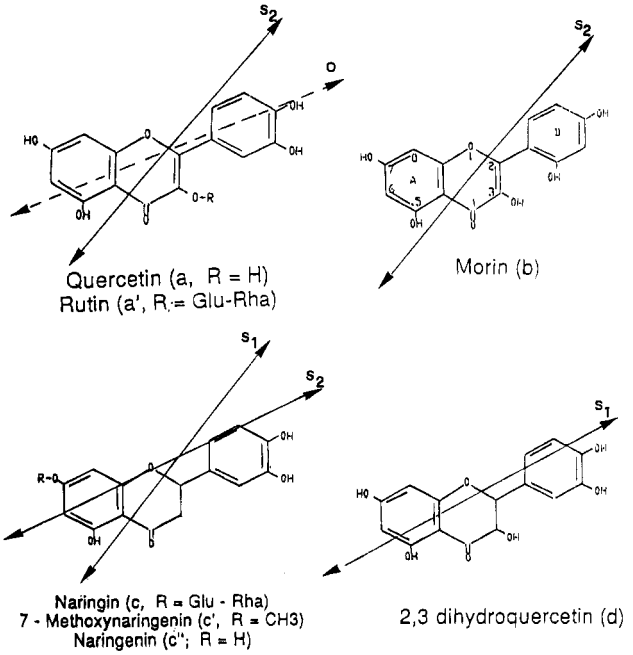
If the orientation of the j direction in the ligand molecular frame is known, or is obtained by independent techniques, S'_j becomes available and the geometry of the binding is obtained. The orientation of the transition moments j of flavonoids **(a–d)** in their chromophoric frames were independently investigated by the liquid crystal linear dichroism (LC LD) technique and by molecular orbital (MO) calculations.

LIQUID CRYSTAL LINEAR DICHROISM (LC LD) TECHNIQUE

This approach to study polarizations of electronic transitions combines the orienting properties of liquid crystalline solvents with the high sensitivity of the modulated techniques to record LD spectra (Samorì, 1983, 1988a). Both lyotropic (Samorì and Mattivi, 1986; Laurent and Samorì, 1987) and thermotropic (Samorì, 1988a) liquid crystalline solvents transparent to UV light down to 200 nm and able to dissolve both lipo- and hydrophilic molecules are available. The mesomorphic solutions can be oriented by a magnetic field or by surface treatments. The guest molecules under investigation are forced by the orientation of the solvent to assume a linearly anisotropic orientational distribution which allows their LD spectra to be recorded.

Elongated molecules tend to align their longest axis to the long axes of the rodlike orienting host molecules (Thulstrup and Michl, 1982; Samorì, 1988a) unless specific interactions between guest and host groups overcome and twist off this orientation (Samorì and Mattivi, 1986). The long axis of the flavonoid aromatic

Chart 1. Formula of the Investigated Flavonoids[a]



[a] The expected axis (o) of preferential alignment to the optical axis of the host liquid crystalline solvent is shown with a dashed line (- - -). This solvent orients guest flavonoid molecules and makes it possible to record their linear dichroism by the liquid crystal linear dichroism technique (LC LD). The directions of polarization of the electronic transitions to the lowest energy states S_1 and S_2 computed by molecular orbital calculation are also reported.

chromophore is expected to be that which is preferentially aligned to the sample director: we call it the orientational (o) axis of the molecule (Chart 1). Positive LC LD signals should therefore be obtained (in eq 1: $OD_{||} > OD_{\perp}$) for transitions polarized on the chromophore plane along this (o) axis or along directions very close to it. Negative LC LD bands should be instead recorded for transitions perpendicularly polarized. We will see that in the LC LD spectra of flavonol and flavanone chromophores the band responsible for their flow LD spectra is positively signed.

RESULTS

Flow Linear Dichroism. The flow LD of flavonols **a**, **a'**, and **b** and flavanones **c** and **d** are reported with their relative average absorption spectra in Figure 1. Quercetin is practically insoluble in water. We were therefore obliged to record its flow LD spectrum in buffer–30% ethanol (Figure 1A). We recorded the spectrum of **b** also in this medium in order to link together the data recorded in buffer and buffer–ethanol. Figure 3 shows the titration curves obtained at the same DNA concentration (10 mg/mL) by recording the LD of flavonols when their concentrations were ranging from 0.1 to 1.0 mM. This range was limited by ligand solubilities. The LD signals of flavanones were too low for obtaining their titration curves.

We also investigated the LD profile of the DNA band centered at 260 nm upon addition of the investigated flavonoids. No significant modifications of the LD spectrum of DNA in this region was caused by flavonoid binding. No evidence of changes in the hydrodynamic behavior of the DNA chains, i.e. in their flexibility and in their secondary structure conformation was thus obtained by this technique.

The flavonoid flow LD spectra in Figure 1 are characterized by the negative band centered at about 380 nm (flavonols, Figure 1A–C) or 330 nm (flavanones, Figure 1D,E). The band edge of the DNA absorption shows that also the 260 nm band of the bases has, as expected (see supra), a negative LD. The intensities of the LD spectra of the different flavonoids are very different and that of **d** is very close to zero. The interpretation of these spectra requires information about the transition polarizations which can be obtained by molecular orbital (MO) calculations and LC LD spectra.

Molecular Orbital Calculations of the Electronic States and Liquid Crystal Linear Dichroism Spectra. Quantum mechanical MO calculations on flavonol and flavanone chromophores were carried out using the CNDO/S method (Del Bene and Jaffè, 1968). The energies and the oscillator strengths of the most strongly allowed and lowest energy calculated electronic transitions of **a'** (where the glycosyl residue was replaced by a methyl group) **b**, **c'**, and **d** fit the spectra of these compounds well.

The flavonol excited states derive essentially from an electron promotion which is localized on the benzopyran-4-one part of the molecule and are basically polarized in the benzopyran-4-one plane. Chart 1 reports the polarization directions on the molecular plane of the lowest energy allowed transitions to S_1 or S_2. Other intense $\pi\pi^*$ states were calculated at much higher energy (>210 nm) and did not appear relevant to the problem under examination.

As a result of hydrogen addition to the C_2–C_3 double bond, compound **c** presents the first singlet transition at higher energy than those of **a** and **b**. The most intense state is now the second S_2. The former transition to S_1 can account for the lower energy shoulder centered at about 340 nm on the DNA band edge. This shoulder characterizes the flow LD spectrum of **c** (Figure 1D). Calculations provide evidence that this band is polarized in the xy plane along the direction reported in Chart 1.

The calculations predict that the polarization direction of the transition to the first singlet state S_1 of **(d)** is tilted 17° over the benzopyran-4-one plane and its projection on that plane is reported in Chart 1. This is the lowest energy most intense transition and it is likely to be responsible of the absorption band of **d** at about 320 nm (Figure 1E).

These CNDO predictions are basically supported by the LC LD spectra reported in Figure 2. In both spectra the lowest energy bands of the two chromophores are positively signed. As shown in the introductory section on this technique this sign indicates that the polarizations of these bands are along directions which lay on the chromophore plane or very closely.

The interpretation of the flow LD spectra can thus rely on MO calculation and LC LD spectra which independently rule out the possibility for the most intense lowest energy transitions of derivatives **a**–**d** to be polarized perpendicularly to the benzopyran-4-one plane. This possibility would overturn our interpretation of the flow LD spectra in terms of binding to DNA by intercalation (see below).

DISCUSSION

Flow LD Indicates that the Investigated Flavonoids Can Intercalate DNA. The non-null flow LD signals exhibited by **a**, **a'**, **b**, and **c** in correspondence of their lowest-energy absorption bands (Figure 1A–C) detect and reveal that those compounds bind DNA. We

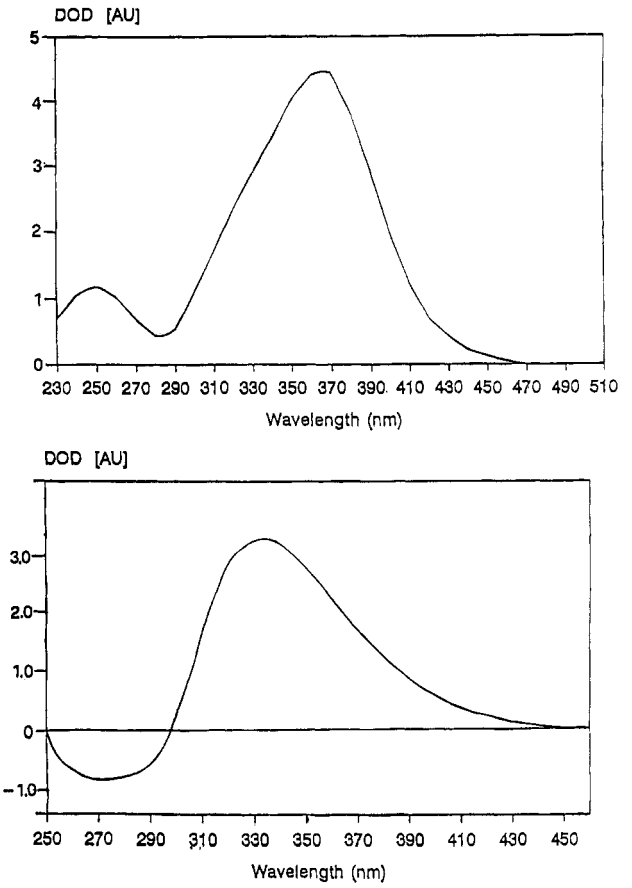


Figure 2. Profiles of the liquid crystal linear dichroism spectra, reported in arbitrary units (AU): (upper) morin (**b**), (lower) naringenin (**c'**).

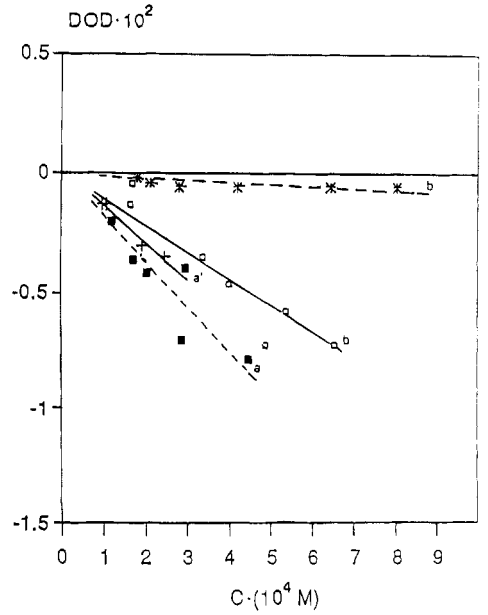


Figure 3. Flow LD vs total ligand concentration titration curves (best fitting) in buffer (Tris 100 mM, NaCl 200 mM, pH 7.5) (—) and buffer–EtOH 30% (- - -) of **a** (■), **a'** (+), and **b** (□ *). The curves are traced up to the solubility limit we found for the different compounds.

tried to independently characterize this DNA–flavonoid binding by an equilibrium binding analysis based on the dextran–polyethylene glycol (PEG)–water two-phase system (Norden et al., 1978; Norden and Tjerneld, 1982). The sensitivity of this analysis was not high enough to reveal the affinity of these flavonoids toward DNA which had been instead clearly displayed by the much more sensitive flow LD technique.

The negative sign of the flow LD signals makes it possible to directly recognize that the flavonoid binding takes place by intercalation. In fact, as shown in the introductory paragraph on the flow LD, a negative sign monitors that upon binding the flavonoid transition moments are settled perpendicularly to the DNA axis. The MO calculations and the LC LD spectra demonstrated that the bands exhibited by the flow LD spectra of **a**, **a'**, **b**, and **c** are polarized on the benzopyran-4-one (flavonol) or 2,3-dihydrobenzopyran-4-one (flavanone) average chromophoric plane. The molecular plane of bound flavonols lies therefore perpendicular to the DNA axis: this corresponds to the intercalation geometry. The flow LD signals of **d** is very close to zero. As we will show at the end of this section, this cannot be ascribed to the 17° tilt over the chromophore plane predicted by the MO calculations for the polarization direction of the transition under investigation but to the inability of **d** to bind DNA.

Flow LD Monitors the Different Binding Affinity of Flavonoids toward DNA. When a binding equilibrium is settled between a ligand and DNA the ligand isotropic absorption comes from the contributions of both its bound and free molecules. The absorption at ∞ or zero DNA concentrations (Abs_1^{∞} or Abs_1^{0}, is due to bound or free molecules, respectively. Between these two limiting conditions

$$Abs_1^{DNA} = f_1^{b} Abs_1^{\infty} + f_1^{fr} Abs_1^{0} \quad (3)$$

where f_1^{b} and f_1^{fr} are the fractions of the bound and free ligand molecules, respectively.

The flow LD is a binding-induced physical property. By this technique we are therefore able to "see" only the bound ligand molecules and at any DNA concentration the LD depends upon the concentration of ligand molecules bound in that condition.

$$LD_1^{DNA} = f_1^{b} LD_1^{\infty} \quad (4)$$

Formulas 3 and 4 are equivalent to formulas 9 and 10 of Norden et al. (1978). From eqs 1 and 2

$$LD_1^{DNA} = 3 f_1^{b} A_{is}^{\infty} S_a S'_1 \quad (5)$$

where A_{is}^{∞} is the isotropic absorption at ∞ DNA concentration (Samorì, 1988b).

We can reasonably assume that both the DNA orientation (S_a) and the orientation of the ligand molecule with respect to the DNA axis (S'_1) does not change within strictly homologous ligands having the same chromophore system. A comparison between their LD signals can therefore provide a direct estimation of f_1^{b}, i.e. of their relative affinities toward DNA.

Intercalation, whenever it takes place, occurs with the molecular plane of the guest molecule perpendicular to the axis of the host DNA chain. Tilting of the long axes of intercalating ligands due to steric overcrowding was in the past suggested, but it was most likely due just to problems in determining the absolute value of the order parameters S_{JJ} (Norden et al., 1992). By Alchemy II we computed the lowest energy conformations of the investigated compounds. Their stereostructures were reported in Figure 4, under the best perspective in order to display their planarity. The intercalation of the planar aromatic rings like those of the flavonols **a**, **a'**

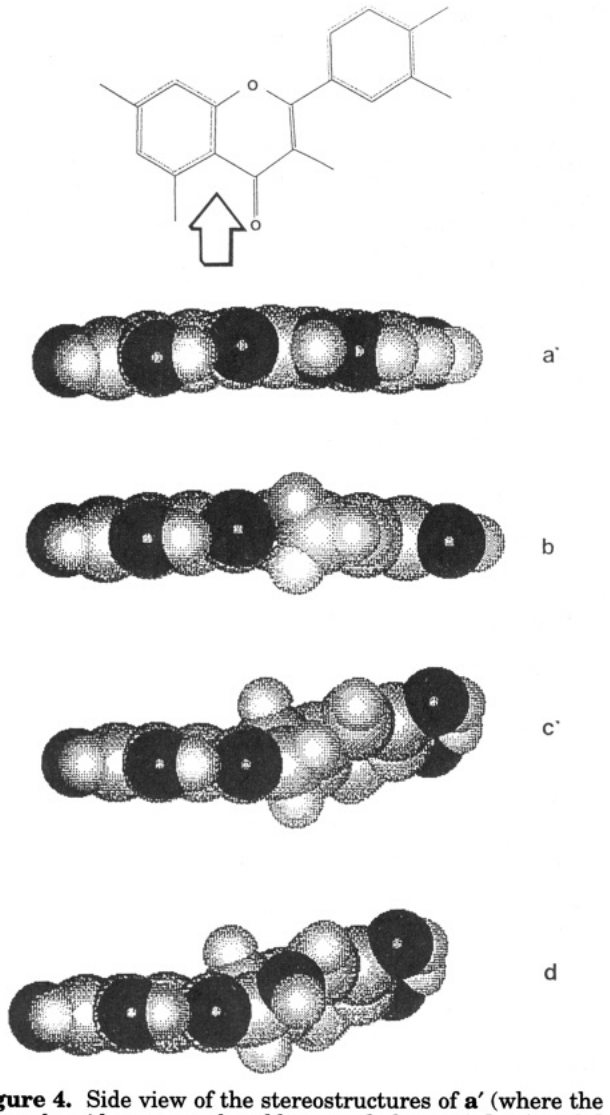


Figure 4. Side view of the stereostructures of **a′** (where the glycosyl residue was replaced by a methyl group), **b**, **c′**, and **d** computed by Alchemy II. The nonplanarity of the flavanone chromophores, **c′** and **d**, and the almost perfect planarity of the flavonols, **a′** and **b**, are well marked.

and **b** (see Figure 4) is thus certainly taking place with the ideal perpendicular geometry. This ideal intercalation geometry is expected to be maintained also when the ligand aromatic system is not planar as in **c** and **d**. Their nonplanarity is not changing the binding geometry: it leads to steric overcrowding which decreases the binding affinity. The intercalation geometry is thus the same for all flavonoids, i.e. they get the same S'_1. We can therefore infer the sequence of affinities of the investigated flavonoids for DNA from the intensities of their flow LD spectra in Figure 1 and the plot in Figure 3. This plot clearly shows that the affinities of **a′** and **b** in buffer are very close to each other and similar to that exhibited by **a** (insoluble in H_2O) in 30% ethanol. In this latter solvent **b** exhibited a tendency to bind DNA much lower than that of **a**. These data suggest that the affinities of planar flavonols are in sequence **a** $\gg$ **a′** $\cong$ **b**. The intensities of the LD signals of **c** and **d** are at least 1 order of magnitude smaller than those of flavonols **a** and **b** (see Figure 1) at the same isotropic absorption. This can be ascribed to the overcrowding due to the nonplanarity of their aromatic system which reduces their ability to intercalate DNA. The almost null ability of **d** is inferred from its almost null flow LD spectrum (Figure 1E) which cannot be ascribed to the out-of-plane tilt of 17° of the polarization ($\alpha_2 = 73°$) predicted by the MO calculations. In fact this tilt can affect, by only about 30% the flow LD intensity which would correspond to an in plane polarization ($\alpha_1 = 90°$). In fact from eqs 1 and 2 the ratio between the LD intensities corresponding to α_1 and α_2 is given by

$$(S_{jj})_1/(S_{jj})_2 = (S'_1)_1/(S'_1)_2 = (3\cos^2\alpha_1 - 1)/(3\cos^2\alpha_2 - 1) = 1.32 \quad (6)$$

Only an almost null affinity for DNA can therefore account for the almost null flow LD spectrum of **d**.

Conclusion. We demonstrated by the flow LD technique that several of the investigated flavonoids can bind DNA by intercalation. We could not estimate their binding constants by equilibrium binding analysis. The binding constants are certainly very low, because high concentrations of flavonols were required to record flow LD signals and the flow LD technique is highly sensitive. We could infer only the sequence of their relative tendency to bind DNA. Maybe it is fortuitous, but this sequence basically follows that of the biological activity of these flavonoids (Huang and Thomas Ferraro, 1992).

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