SPECTROSCOPIC EVIDENCE FOR THE INTERACTION OF TINGENONE WITH DNA

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1. Introduction

Tingenone (fig.1) is a quinonoid triterpene [1,2], isolated from the bark and roots of various plants of the Celastraceae and Hyppocrateaceae families as a red orange pigment, showing antineoplastic activity [3–5]. This compound, practically insoluble in water, is prevailingly apolar. However, the solubilization of tingenone can be favoured owing to the interactions occurring with more polar compounds, such as sodium deoxycholate or DNA, thus reducing the apolar surface of tingenone in contact with the aqueous environment. Its solubilization in water may be important for a better pharmacological application and, therefore, its binding to sodium deoxycholate in aqueous solution was studied [6]. We have tried to bind tingenone to some DNA species in order to gain information on the possible types of interaction, since the antitumour activity of tingenone could depend on this binding.

To clarify the role of the DNA's base composition, which affects the macromolecular order and stability, three double-helical DNA species (from calf thymus, *Clostridium perfringens* and *Micrococcus lysodeikticus*) were investigated.

Here we present the results of spectroscopic investigations which support the interaction between tingenone and DNA. No evidence could be gained about a relationship between the binding mode and the base composition.

2. Materials and methods

Tingenone, extracted with light petroleum from the ground bark of *Maytenus ilicifolia* and crystallised from ethyl acetate, was repeatedly purified on a silica gel column (70–270 mesh, Macherey Nagel, Düren) with chloroform as eluant. The chloroform was evaporated and the solid residue, dried in high vacuum at 80°C, was kept in the dark. The concentration of tingenone in the DNA–tingenone solutions was determined on the basis of the molar extinction coefficient of tingenone at 425 nm in ethanol–water (50/50, v/v) solution (ϵ_{max} = 11 755 l . mol^{-1} . cm^{-1}) and in ethanol–Tris–HCl 0.01 M (50/50, v/v, pH 7.30) solution (ϵ_{max} = 13 794 l . mol^{-1} . cm^{-1}). These ϵ_{max} values do not change within the range of concentration studied here.

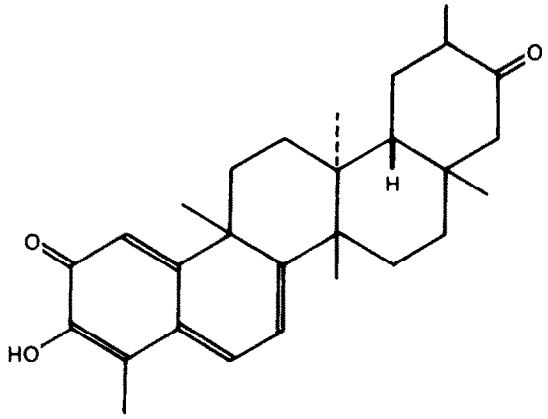

Fig.1. Molecule of tingenone.

Calf thymus DNA (highly polymerized sodium salt, type I), *Micrococcus lysodeikticus* (highly polymerized, type XI) and *Clostridium perfringens* (type XII) were products of Sigma, St Louis, MO. Stock solutions of DNA were prepared in 0.01 M

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Tris–HCl (pH 7.30) and dialyzed *vs* 6 batches of Tris buffer. The DNA concentrations were based on an assumed value for the molar extinction coefficient with respect to nucleotides of 6420 l . mol^{-1} . cm^{-1} for calf thymus at 258 nm [7] and of 6300 l . mol^{-1} . cm^{-1} for *Micrococcus lysodeikticus* [8] and *Clostridium perfringens* [7] at 260 nm and 258 nm, respectively. The aqueous solutions of calf thymus DNA were prepared by dialysis of the buffered solutions. Denatured DNA was obtained by heating a solution of native DNA at 100°C for 10 min and cooling it immediately at 0°C. Doubly glass-distilled water was used throughout.

The binding of tingenone to DNA was achieved as follows. A $1–2 \times 10^{-3}$ M solution of tingenone in ethanol (Uvasol, Merck) was added portion-wise (10 μl) to a cooled (2–4°C) DNA aqueous solutions ($2–2.5 \times 10^{-4}$ M) with or without buffer in such a way as to reach an ethanol/water ratio of 0.03 (v/v). Thus, the solution was shaken at 5°C by using a Griffin flash shaker for 100 h and subsequently centrifuged at 10 000 rev./min at 5°C until the A_{425} remains constant. Lyophilization of the solution and solubilization of the dry product in water or in buffer allowed us to obtain solution without ethanol. The binding of tingenone to DNA depends on the ionic strength. Generally, the binding in a buffered solution is ~4-times lower than in a corresponding unbuffered one. Moreover, the dilution of a solution must be avoided in order to not decrease the tingenone bound to DNA.

Attempts to bind tingenone to DNA by means of the solvent-partition method [9], using chloroform, ethyl acetate and 1-hexanol, were unsuccessful.

Absorption measurements were carried out with a Beckman DK 2A spectrophotometer. Circular dichroism (CD) spectra were recorded on a Cary model 61 spectropolarimeter.

3. Results and discussion

The CD spectra of tingenone, calf thymus, *Micrococcus lysodeikticus* and *Clostridium perfringens* DNA with the range 200–300 nm are shown in fig.2.

Three samples containing tingenone with calf thymus, *Micrococcus lysodeikticus* or *Clostridium perfringens* DNA were prepared, using the same concentration for the 3 DNA species and adding the same amount of tingenone. Tingenone/nucleotide ratios of

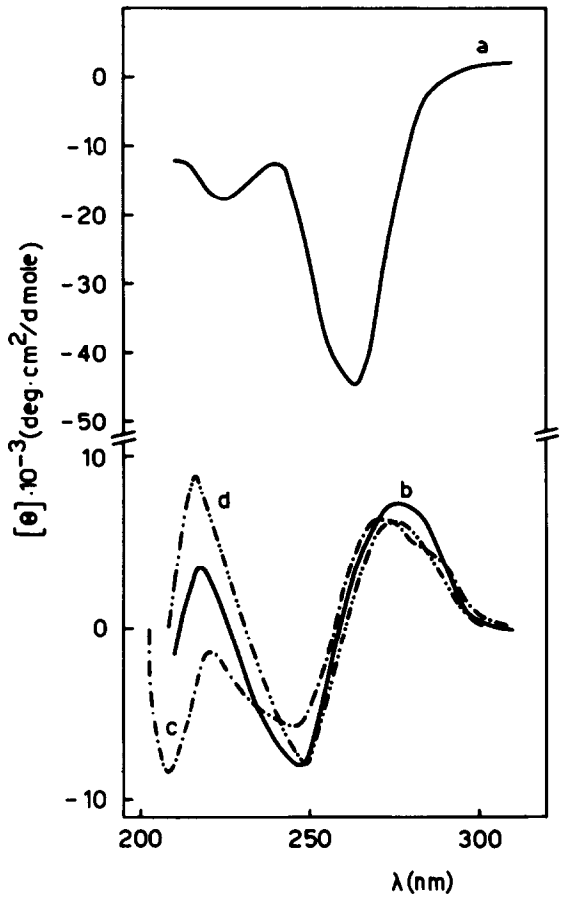

Fig.2. CD spectra of: (a) tingenone 1.9×10^{-5} M, in Tris–HCl 0.01 M–ethanol solution (50/50, v/v) (pH 7.3); (b) calf thymus DNA, (c) *Micrococcus lysodeikticus* DNA, (d) *Clostridium perfringens* DNA, 2.1×10^{-4} M, in Tris–HCl 0.01 M–ethanol solution (97/3, v/v), (pH 7.3).

~0.03, ~0.05 and ~0.07, respectively, were obtained for the 3 DNA species. The corresponding CD spectra, recorded in a region where tingenone absorbs and DNA does not, are reported in fig.3 and show some changes with respect to that of tingenone, mainly near 375 and 460 nm. These changes allow to infer that the interactions between tingenone and DNA affect the optical properties of tingenone and that, probably, the binding mode depends on the DNA species. A sample, formed by tingenone–calf thymus DNA with a tingenone/nucleotide ratio of ~0.08, was prepared by dissolving in Tris–HCl 0.01 M the dry product obtained by lyophilization of an aqueous solution with a 3% of ethanol. Its CD profile (see fig.3 curve e), as compared with curve b, seems to indicate

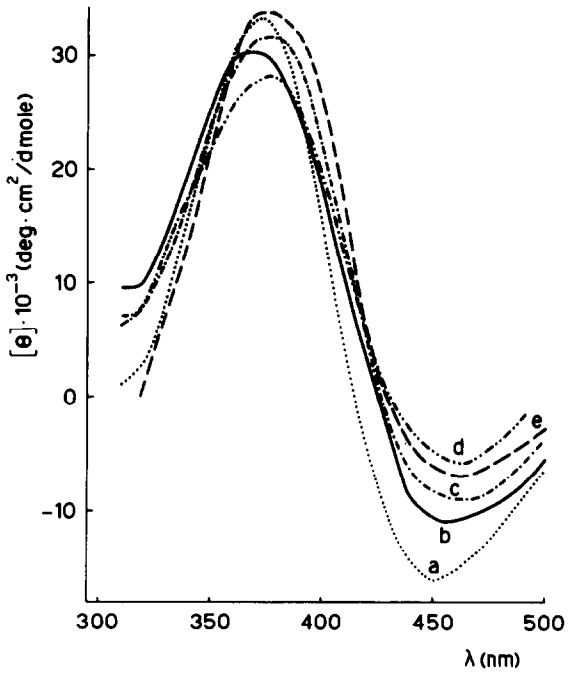

Fig.3. CD spectra of: (a) tingenone; and tingenone with: (b) calf thymus DNA, (c) *Micrococcus lysodeikticus* DNA, and (d) *Clostridium perfringens* DNA in the same conditions as in fig.2. Tingenone is ~6.5 X 10^{-6} M (b), ~1.1 X 10^{-5} M (c), ~1.4 X 10^{-5} M (d), respectively. (e) refers to a tingenone (~1.7 X 10^{-5} M)–calf thymus DNA (2.1 X 10^{-4} M) solution in Tris–HCl 0.01 M (pH 7.3).

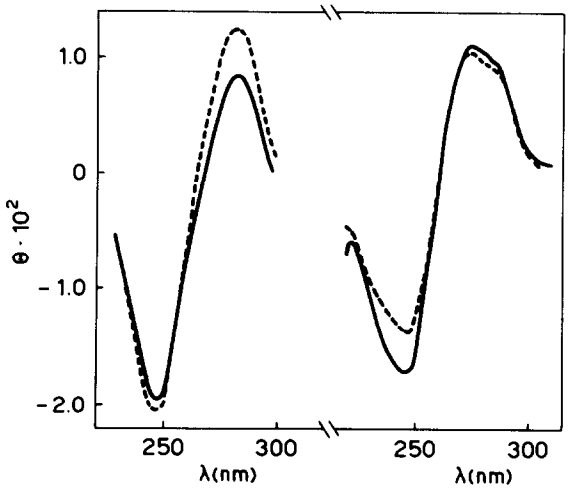

Fig.5. Left: continuation of fig.3e together with the calculated curve (– – –). Right: continuation of fig.3c together with the calculated curve (– – –).

that the type of binding could be influenced by the preparation mode. In fact, the solutions of curves b and a differ only for the presence or less in the solvent medium of a 3% ethanol. Another evidence, supporting the binding of tingenone to DNA, is given in fig.4 where the observed absorption spectrum of a tingenone–*Micrococcus lysodeikticus* DNA solution shows hyperchromicity with respect to the calculated

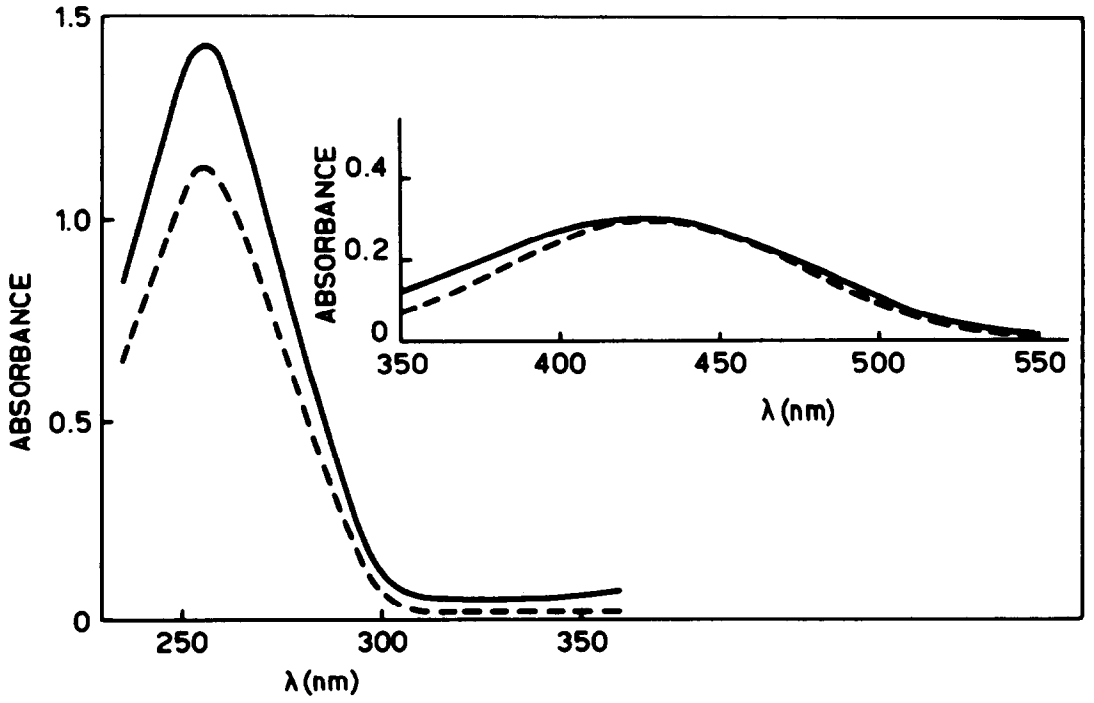

Fig.4. Absorption spectrum of tingenone (~1.1 X 10^{-5} M)–*Micrococcus lysodeikticus* DNA (2.1 X 10^{-4} M) in Tris–HCl 0.01 M–ethanol solution (97/3, v/v) (pH 7.3), together with the calculated spectrum (– – –). Optical path: 1 cm (UV) and 2 cm (visible).

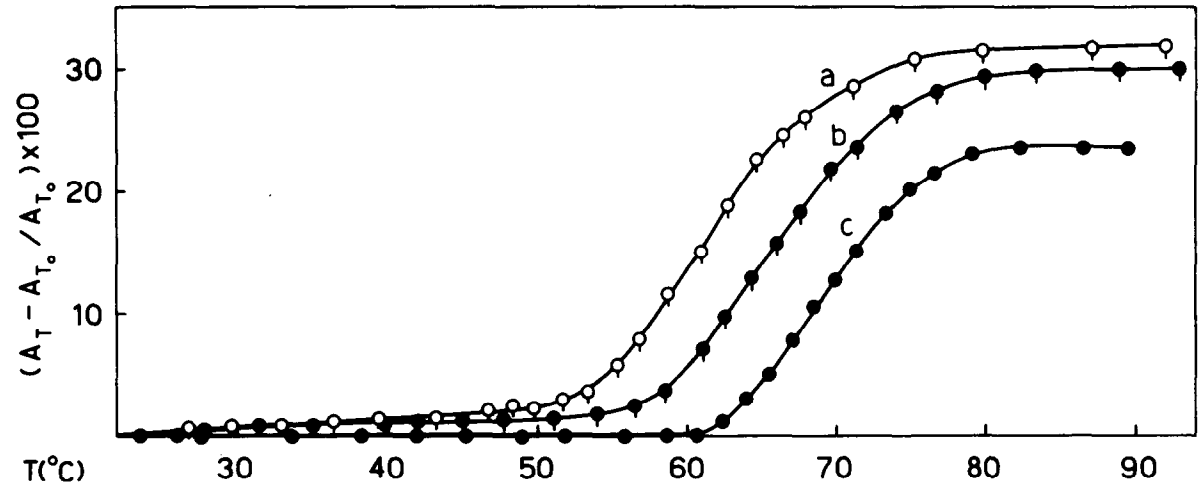

Fig.6. Thermal profiles of calf thymus DNA solutions, obtained from a lyophilized, without or with tingenone in Tris–HCl 0.01 M (pH 7.3): (a) DNA, 2.0 × 10^{-4} M; (b) DNA, 2.0 × 10^{-4} M–tingenone, ~4.3 × 10^{-6} M; (c) DNA, 1.6 × 10^{-4} M–tingenone, ~1.2 × 10^{-5} M.

one of ~27% at 260 nm. Less remarkable differences are displayed by the other DNA species in the CD and UV spectra. In addition, the wavelength region more influenced by the binding of tingenone is near 280 nm for calf thymus and *Clostridium perfringens* DNA and near 248 nm for *Micrococcus lysodeikticus* DNA (see fig.5), so that it is reasonable to suppose that the DNA structure and composition affect the type of interaction between DNA and tingenone.

Unfortunately, it seems that no clear evidence exists between the amount of tingenone linked to DNA and the base content on the basis of the tingenone/nucleotide ratio observed in many samples prepared as previously described.

The binding of tingenone to DNA improves its stability increasing the melting temperature (T_m) as is shown in fig.6,7, where the hyperchromicity $(A_T - A_{T_o}/A_{T_o}) \times 100$ *vs* the temperature is reported.

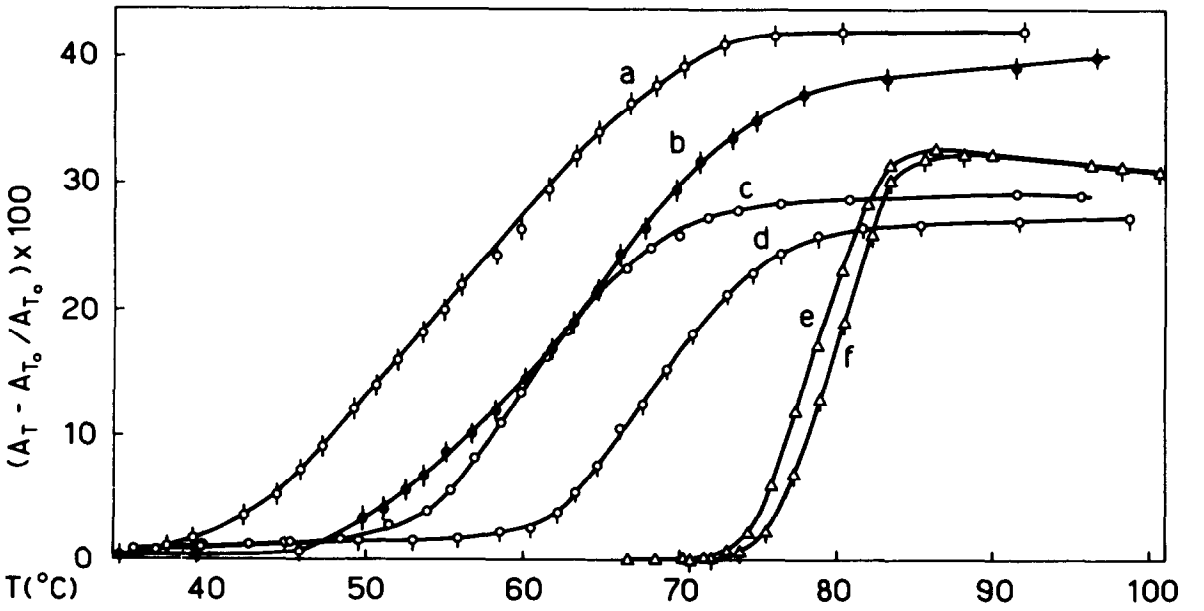

Fig.7. Thermal profiles of calf thymus and *Micrococcus lysodeikticus* DNA solutions (pH 7.3) containing 3.1% ethanol: (a) calf thymus DNA, 2.2 × 10^{-4} M, in Tris–HCl 0.001 M; (b) as (a) plus tingenone, ~5.2 × 10^{-6} M; (c) calf thymus DNA, 1.5 × 10^{-4} M, in Tris–HCl 0.01 M; (d) as (c) plus tingenone, ~2.2 × 10^{-6} M; (e) *Micrococcus lysodeikticus* DNA, 1.4 × 10^{-4} M, in Tris–HCl 0.01 M; (f) as (e) plus tingenone ~3.5 × 10^{-6} M.

A_T and A_{T_0} are the A_{260} of a solution at a given temperature and at a reference temperature (25°C), respectively. In this connection it must be noticed that the absorbance of tingenone could not be subtracted, since this compound is insoluble in the solvent media of the solutions investigated. However, the A_{260} of tingenone in ethanol–Tris–HCl 0.01 M (50/50, v/v) (pH 7.3) remains constant within the range 25–75°C. T_m, calculated as half of the maximum value of the hyperchromicity, rises from 61.2–65.6 and 69.4°C by increasing the tingenone/nucleotide ratio. The same trend can be inferred from inspection of fig.7. Moreover, a better stabilization is achieved when the ionic strength, due to the buffer, increases, passing from curves a and b to c and d and from e to f.

On the other hand, in the case of *Micrococcus lysodeikticus* DNA, which is more stable than calf thymus DNA and presents a higher T_m (78.5°C), the improvement on T_m is much lower (+1.3°C), even if the tingenone/nucleotide ratio is greater than that of the corresponding calf thymus DNA solution of fig.7. This may be interpreted in terms of a binding which induces ordering in some disordered segments for calf thymus DNA, whereas no change of the DNA structure occurs for *Micrococcus lysodeikticus* DNA.

Experiments of binding of tingenone to denatured calf thymus DNA led to a very low complexation ratio (at most 0.013) and no spectroscopic and renaturation effects were observed.

The size and the shape of tingenone molecule are favourable for its inclusion in the narrow groove of DNA and, furthermore, hydrogen bonds can be formed between the hydroxyl group of tingenone and the phosphate group of DNA. Since tingenone is a bulky molecule, an intercalation mechanism seems to be unlikely, unless only the nearly planar A and B rings are engaged.

Acknowledgement

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